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Co-delivery of Indoleamine 2,3-dioxygenase prevents loss of expression of an antigenic transgene in dystrophic mouse muscles

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Abstract:

A significant problem affecting gene therapy approaches aiming at achieving long-term transgene expression is the immune response against the protein product of the therapeutic gene, which can reduce or eliminate the therapeutic effect. The problem is further exacerbated when therapy involves targeting an immunogenic tissue and/or one with a pre-existing inflammatory phenotype, such as dystrophic muscles. In this proof-of-principle study we co-expressed a model antigen, bacterial β-galactosidase, with an immunosuppressive factor, indoleamine 2,3-dioxygenase 1 (IDO1), in muscles of the *mdx* mouse model of Duchenne muscular dystrophy. This treatment prevented loss of expression of the transgene concomitant with significantly elevated expression of T regulatory (Treg) markers in the IDO1-expressing muscles. Moreover, co-expression of IDO1 resulted in reduced serum levels of anti-β-gal antibodies. These data indicate that co-expression of genes encoding immunomodulatory enzymes controlling kynurenine pathways provide a viable strategy for preventing loss of transgenes targeted into dystrophic muscles with pre-existing inflammation.

Introduction

A common problem in the development of gene therapy for inherited deficits is the risk of immune responses against the re-expressed therapeutic protein. Such protein, due to the total absence of its endogenous expression or because of differences with the endogenous mutant gene product, becomes a neo-antigen. The transgene-expressing cells also become the immune response target, which leads to the cessation of the therapeutic effect and often to further tissue damage (1, 2). The consequences to the recipient can range from transient without significant impairments to severe and life threatening. Many factors affect the immunogenicity of therapeutic proteins. Some of these are protein-related, e.g. dose and antigenicity. Others are disease/tissue - related, e.g. the underlying immune status and the target tissue promoting immune responses. Skeletal muscle is an immunogenic location (3) and muscle diseases are

55 often associated with chronic sterile inflammation, which exacerbates the highly unfavorable
56 environment.
57 Duchenne muscular dystrophy (DMD) is an inherited muscle-wasting disease (4) with
58 inflammation (5) leading to severe disability and death of young men. Currently, no treatment
59 improves the long-term outcome. Unfortunately, there is evidence that immunogenic epitopes
60 resulting from new therapeutic modalities aiming at restoration of dystrophin expression (6-9)
61 can result in treatment failure in both experimental and clinical trials (10). Therefore,
62 prevention/suppression of immune responses is pre-requisite for successful therapies. While
63 broad immunosuppression has been shown to prolong transgene expression (11, 12), it
64 disrupts immune responses against pathogens, which is not without risks in already heavily
65 handicapped patients. In contrast, development of methods to induce tolerance to a therapeutic
66 protein would provide a significant advancement in gene therapy.
67
68 While expression of protein in a naïve organism usually causes immunisation, it may also lead to
69 the development of the immune tolerance. Mechanisms causing the switch between T cell
70 response and unresponsiveness include T cell anergy, clonal deletion of antigen-specific T
71 lymphocytes and regulatory T cell (Treg) activation (13). Whether a T cell becomes an effector
72 cell or is tolerised depends on the status of antigen-presenting cells (APCs) that interact with it
73 (14-18). This activation status of APCs is, in turn, dependent on the inflammatory signals
74 provided by tissue damage, cytokine content, gene therapy vector and inflammatory signals.
75 The catabolism of the essential amino acid L-tryptophan by indoleamine 2,3-dioxygenase (IDO)
76 and tryptophan 2,3-dioxygenase (TDO) enzymes produces kynurenines, which regulate innate
77 and adaptive immune system and maintain tolerance to “self” antigens (19). IDO1 plays a key
78 role in fetal-maternal tolerance, and animals exposed to antigen neonatally were found unable
79 to mount humoral or cytotoxic responses when re-challenged with DNA constructs encoding
80 such antigens as adults (20-23). Importantly, there is evidence that IDO1 induces peripheral
81 tolerance and the retro-control of immune responses (24).

We provide a proof-of-principle that an approach co-triggering the kynurenine mechanism prolongs expression of a model antigenic transgene in adult dystrophic *mdx* mouse muscles. Importantly, the IDO does not need to be targeted to specific cells.

Results & Discussion

To minimise the confounding immunizing and immunomodulatory effects of viral vector proteins we used intramuscular targeting of plasmid vectors expressing bacterial β-galactosidase (lacZ gene) model antigen alone or co-expressed with mouse IDO1.

Engineering, expression and characterisation of vectors

The choice of the promoter/enhancer elements controlling the transgene can seriously affect its immunogenicity. The ubiquitously-active control elements (e.g. CMV) trigger immune responses because transgenes become expressed directly in the APCs, which results in increased antigen presentation (25) and rapid recruitment of cytotoxic T cells (CTLs). Tissue-specific promoter/enhancer control can improve the duration of transgene expression. Nevertheless, for many transgenes, immune responses (albeit humoral, of lower magnitude and delayed in comparison with the CMV-driven construct) are still being triggered because proteins released from cells are processed and presented by APCs, which causes activation of T-helper cells and ultimately the response. Therefore, to maximise the immunogenic effect, we used the CMV promoter. For tolerance induction we generated two mouse IDO1-expressing plasmids: pcDNA3.CMV.mIDO1 driven by the same ubiquitously-active CMV and pcDNA3.CD11c.mIDO1 controlled by the minimal CD11c promoter shown previously to be active primarily in dendritic cells and macrophages (25-27). The pcDNA3.CD11c.mIDO1 was made by excision of the CMV promoter using *Mfe*I and *Acc*65I and ligation of the fragment containing the 760 bp mouse CD11c minimal promoter region. The expression of IDO1 from these constructs was assessed following transfection of SC5 dystrophic myoblasts (28), HEK-293 cells or RAW264.7 mouse

macrophages *in vitro* and Western blotting confirmed IDO1 protein being expressed in transfected HEK293 cells (Fig 1A). The same approach was used to confirm expression of the pcS2+β-gal plasmid (Fig 1B).

Co-administration of IDO1 plasmid reduces loss of β-gal *expression in skeletal muscles in vivo.*

We targeted β-gal into *Tibialis anterior* muscles of 4 week old male *mdx* mice as, at this stage, the *mdx* muscle has all the hallmarks of the disease with its highly inflammatory environment. β-gal expression was initially analysed 7, 14 and 21 days following a single i.m. plasmid injection. This time-course analysis revealed that β-gal was readily detectable in injected muscles at 7 days. Protein expression was decreased at 14 and undetectable at 21 days (Fig 2A). Subsequently, this plasmid dose was injected alone (control) or co-injected with one of the IDO1-expressing plasmids and β-gal expression was analysed at 14 days (Fig 2B-2D).

Western blotting analysis of muscle extracts revealed that only 25% of muscles injected with the lacZ plasmid alone had detectable β-gal protein expression 14 days post-injection. In contrast, 69% of muscles co-injected with pcDNA3.CMV.mIDO1 plasmids showed detectable β-gal expression while co-injection with pcDNA3.CD11c.mIDO1 plasmid (targeting IDO1 to APCs) resulted in protein being expressed in 42% of muscles (Fig 2B). Therefore, IDO1 does not need targeting to APCs. At the equal dose, the qPCR analysis (Fig 2C) demonstrated that the individual expressions of the lacZ transcript relative to GAPDH endogenous control were higher in the majority of co-injected samples but the averages did not differ significantly due to highly variable expression levels and the relatively low sample number. However, injection of double the amount of β-gal plasmid in the control group resulted in statistically significant reduction rather than increase of this protein expression, thus confirming that higher β-gal expression in co-injected muscles was due to the IDO1 immunosuppression (Fig 2D). The lacZ expression heterogeneity was confirmed in the muscle sections stained with X-gal: Some of the fibres were strongly labelled while others were expressing very low transgene levels (Fig 3). This was in

agreement with previous studies using injection of naked lacZ plasmids into skeletal muscles (29).

IDO1 expression changes the inflammatory profile in injected muscles

Analysis of serial transverse sections of TA muscles showed inflammatory cells to be surrounding and infiltrating both the highly- and the weakly-expressing fibres (Fig 3). There was no significant difference between samples injected with β-gal or co-injected with IDO1 plasmids. The immunohistochemical analysis of markers for the specific inflammatory cell subpopulations infiltrating the tolerised vs. non-tolerised muscles was inconclusive (data not shown).

To distinguish the exogenous from the endogenous IDO1 expression we used RT-PCR with primers detecting the plasmid-driven transcript only (Fig 4A). Having confirmed the exogenous IDO1 expression, qPCR was used to establish the relative levels of IDO1 mRNA and that confirmed a statistically-significant increase in the co-injected muscles (Fig 4B).

The qPCR analysis of several key marker genes for different T cell subsets showed statistically significant increase in the expression of Cd4, Cd8 and forkhead box P3 (Foxp3) genes in muscles co-injected with IDO1 plasmids (Fig 5). This pattern is interesting as increased expressions of lymphocyte markers were concomitant with higher levels of Foxp3. This transcription factor regulates the development and function of T-regulatory (T-reg) cells (30) and therefore overexpression of IDO1 appears to prolong antigen expression *via* the induction of T-regs.

The levels of interleukin 10 (Il10) and Il12a expression did not show statistically significant differences in these samples (Fig 5). Previous studies demonstrated regulatory interactions between IDO1, IL-10 and IL-12 in antigen presenting cells (31, 32) and IDO1 suppresses the IL-10, as kynurenic acid reduces IL-10 production (33). Interestingly, the decrease in expression of IL-10, which is an anti-inflammatory cytokine (34, 35) and one of the mediators of T-reg functions, did not reach statistical significance in IDO-1-expressing muscles. Similarly IL-12,

158 which is involved in the development of Th1 $CD4^+$ T cells, which leads to cytotoxic T-lymphocyte
159 activation (36) and also activate natural killer (NK) cells (37) was not significantly reduced,
160 although its levels were consistently lower in IDO1-injected muscles. Therefore, the IL-10/IL-12
161 regulatory interplay may still have a role in the local microenvironment but changes may not be
162 detected when analysed globally in an otherwise inflammatory environment of the dystrophic
163 muscle.
164

165 Finally, the ELISA performed to quantify the serum anti-β-gal antibody levels showed
166 significantly lower response in animals co-administered with the IDO1 plasmid (Fig 6). This is
167 consistent with the studies in haemophilic mouse models, which revealed that kynurenins
168 prevented generation of anti-FVIII antibodies and suppressed FVIII-specific B cells by a
169 mechanism involving the IDO1-dependent induction of T-regs (38). Interestingly, a recent study
170 demonstrated a novel role for IDO1, intrinsic to B cells, where it acts as a regulator of humoral
171 immunity (39). Together, these and our findings indicate that IDO1 strategies for preventing
172 immunisation in gene therapy approaches should be explored while further studies are needed
173 to help our understanding of the exact mechanism(s) involved.

174 In summary, this proof-of-principle study was performed to demonstrate that local expression
175 of even strong non-self antigens in tissues with pre-existing inflammation can be prolonged
176 using enzymes modulating kynurenine pathways. These data point towards a viable strategy for
177 preventing transgene loss in various gene therapy settings. Recently, this approach was used to
178 induce tolerance to transplanted organs, thus reducing need for immunosuppressive treatments
179 (40).
180

181 **Materials and Methods**

The *mdx* C57Bl/10ScSn-Dmdmdx/J 4 week old male mice were used in accordance with the institutional Ethical Review Board and the Home Office (70/7549) approvals.

The lacZ (pcS2+Cβ-gal) and the primary pcDNA3.IDO1 plasmids were obtained via collaboration (see Acknowledgements).

The following antibodies were used: anti-βgal (ThermoFisher Scientific, A11132), anti-IDO clone 10.1 (Merck Millipore; 05-840), anti-actin (A2066) and anti-GAPDH (G9545, both Sigma).

RT-PCR and qPCR analyses

Total RNA was extracted using RNeasy kit (Qiagen) as per manufacturer's instructions. 1 µg of RNA was used for cDNA synthesis and 100 ng of cDNA in RT-PCR. Identification of the exogenous (plasmid-driven)IDO1 was performed using primers: Fv 5'-GAGGCTGGCAAAGATCTCCTGC -3' and Rv 5'- GCTCGAGCGGCCGCCTA-3' in 35 cycles: 94°C; 60°C, 72°C for 1 minute each, followed by a final 9 min elongation step at 72°C.

Quantitative PCR analyses of Cd4, Cd8, Foxp3, Il10 and Il12 transcripts were performed using 25-50 ng of cDNA with Taqman probes (Applied Biosystems) and ViiA™ 7 Real-Time PCR System (Life Technology, UK). All individual muscles were analysed in triplicate and all samples run on the same plate to avoid inter-run variation. Gapdh was previously determined to be the most stably expressed gene in dystrophic muscles (41) and thus used as reference to establish individual gene expression values ($2^{-\Delta\Delta CT}$).

Construction of cd11c.IDO1 expression vector

The minimal functional region of the 5.3 kb CD11c promoter (25, 26) was amplified from the pBluescript.CD11c.Beta Globin Ova I plasmid (gift from Dr. Thomas Brocker) using adapter primers Fv 5'- TCAACGCGTCAATTGAGTATTCTCTTGACCTTGGCTGCC -3' and Rv 5' -

TGAGGTACCGACTGGAGAACAGAAGCAGGC- 3' with MfeI and Acc65I restriction sites for 35 cycles (94°C, 67°C and 72°C for 1 minute each) followed by the final elongation step at 72°C.

The 760 bp amplicon was isolated from an agarose gel, purified and cloned into pcDNA3.CMV.mIDO1 digested with MfeI and Acc65I that removed the CMV promoter cassette, resulting in the pcDNA3. CD11c.mIDO1 plasmid. Positive clones were verified by DNA sequencing. High-purity, endotoxin-free plasmids were made using Endofree Mega Kit (Qiagen, UK).

In vitro cell transfection

HEK-293 and SC5 myoblast cells were authenticated (Promega, and PowerPlex® 16 HS and MycoAlert™ PLUS Mycoplasma Detection Kit, Lonza) and cultured as previously described (28). 24h before transfection 250,000 cells were plated in 35 mm dishes. 2 µg of endotoxin-free plasmid was diluted in Knockout-DMEM (Thermofisher, UK) and complexed with Lipofectamine, GeneCellin (BiocellChallenge), FuGene HD (Promega, UK) and K2 (Biontex) at 37°C for 15 min. The transfection reagents were dispersed and cells grown for 24h, transfection mixtures replaced with the complete medium: Knockout-DMEM with serum (Thermofisher) and total proteins extracted after 72 h.

Analysis of transgene expression in vivo

Plasmids were diluted in 0.9 % saline. For the co-injection experiments, the lacZ plasmid was mixed 1:1 (molar ratio) with either pcDNA3.CMV.mIDO1 or pcDNA3.CD11c.mIDO1. Anaesthetized *mdx* mice were allocated randomly to the experimental and control groups and injected aseptically into both *Tibialis anterior* muscles with 25 µl/muscle (1µg/µl) using the 27G1/2 needle fitted with a plastic collar to maintain depth of injection constant (42). At specific times post-injection mice were killed by CO_2 inhalation and muscles flash-frozen in liquid nitrogen. Blood was collected and serum prepared using a standard method (41).

Protein extraction

Frozen TA muscles were crushed in liquid nitrogen and tissue powders re-suspended in 200 µl ice-cold extraction buffer [1 x cOmplete Lysis M (Roche), 1x cOmplete ULTRA Mini EDTA-free protease inhibitor cocktail tablet, 1 x PhosSTOP phosphatase inhibitor cocktail tablets (Roche)]. Samples incubated on ice for 30 min with occasional vortexing were homogenised further by passing through 27½G needle (3X), centrifuged at 16,000 g for 5 min and protein concentrations in supernatants measured using the Bicinchoninic acid kit (Sigma, UK).

Western Blotting

Protein (40-60 µg) was resolved on SDS-PAGE gel and blotted onto Hybond-P membranes (GE Healthcare). Blots were blocked in 5% w/v non-fat milk in TBST for 1 h prior to probing with a primary antibody (overnight at 4°C), washed with TBST (10 min, 3X) and incubated with the appropriate HRP-conjugated secondary antibody for 1 h at RT. Specific protein bands were visualized using Luminata Forte or Crescendo chemiluminescent substrates (Merck Millipore), images obtained using G-Box (Syngene). Densitometric analyses of protein bands were made using exposure times within the linear range and the integrated density measurement function of Fiji software (43).

Histological muscle analyses

5-10 µm thick muscle cryosections were fixed in 4% w/v paraformaldehyde solution, washed in PBS and stained for 3 days at 37°C in PBS with 500 µg/ml X-gal (Promega), 5mM Potassium Ferricyanide (III), 5mM Potassium Hexacyano Ferrate (II), 2mM Magnesium chloride (all Sigma) and 0.02% Nonidet P40 (Calbiochem). Sections were washed with TBST, counterstained with H&E and images captured using Axiophot (Zeiss) microscope.

ELISA

Serum antibody levels were measured as described previously (1, 44). 96 well Maxisorp® plates (Nunc) were coated with 100 µl/well βgal (Roche) at 10 µg/ml in bicarbonate buffer (pH 9.6) overnight at 4°C, washed with TBST and incubated at RT for 1h with 200 µl/well of blocking buffer (2% Tween 20 in PBS without Ca^{2+}/Mg^{2+}). 100 µl/well of serum (1:100-1:5000 in PBS, 2% Tween 20; analysed in triplicate) or, for standard curve, mouse anti-βgal antibody (1:200-1:40,000; Sigma) were incubated at RT for 2 h. After washing, samples were incubated with goat anti-mouse HRP antibody (1:5000; Sigma) for 2 h at RT in the dark, washed and incubated with TMB substrate (BD Biosciences) at 37°C for ~10 min. The reaction was stopped with 2M H_2SO_4 and read at 450/550nm in a plate reader (Dynex MRX TC II) against the standard curve.

Statistical analysis of the data

All experiments were repeated at least 3 times. The data are presented as means ± SEM. All results were analysed using student T-test or ANOVA with Tukey's Multiple Comparison post-hoc test and $P<0.05$ was considered as statistically significant.

Figure legends

Figure 1

Characterisation of expression vectors *in vitro*. (A) Representative western blots showing a 42kDa IDO1 band in transfected cells. 1 and 2 denote expression in dystrophic SC5 myoblast transfected with Lipofectamine or GeneCellin. Positive control (PC) denotes protein from HEK-293 cells transfected with plasmids driven by the CMV promoter (B) Western blot showing a 120kDa band of β-gal expressed in HEK-293 cells (PC) and in myoblasts (1-4) transfected with different reagents (1 and 2: GeneCellin, 3: K2, 4: FuGene HD). LC denotes the protein loading control and B a blank lane.

Figure 2

Continued muscle expression of β-gal following co-injection with IDO1. (A) Kinetics of β-gal expression in mdx mouse muscles *in vivo*: Protein levels were analysed by Western blotting 7, 14 and 21 days post-injection into *Tibialis anterior* muscles. There was a complete lack of β-gal expression at 21 days. PC represents the positive control using protein expressed in HEK-293 cells and LC is the protein loading control. (B) Graph representing the numbers of β-gal positive TA muscles 14 days post-injection, as assessed by Western blotting, demonstrating the significantly increased numbers of expressing muscles that were co-injected with CD11c-IDO1 plasmid [5 out of 12 muscles (41.67%)] and greater still for CMV-driven IDO1 plasmid [11 out of 16 muscles (68.75%)] compared to only 4 out of 16 muscles (25%) in the control group. (C) qPCR analysis: Individual values plot of the relative expression levels ($2^{-\Delta\Delta CT}$) of β-gal in muscles 14 days post-injection of plasmids (n=5, P=0.068). (D) β-gal protein levels in Western blots comparing double the amount of plasmid in the control (BG) group (n=14, **P=0.008).

Figure 3

Heterogeneity of β-gal expression in injected muscles. (A) X-gal staining shows highly variable protein expression in a small number of fibres (arrows) per area, with no clear correlation between inflammatory cell numbers infiltrating positive fibres in muscles injected with lacZ plasmid only (A,C,D) or co-injected with IDO1 (B,E,). Scale bars = 70 μm.

Figure 4

Expression of exogenous IDO1. (A) Agarose gel electrophoresis of exogenous IDO1 amplification products. The expected 400 bp band is found in RNA samples from co-injected muscles (1-6) but not in the muscle sample injected with lacZ only (β-gal) or in the negative control (nc). NEB quick load 100 bp DNA ladder was used. (B) Individual values plot of the qPCR analysis showing significantly higher expression levels ($2^{-\Delta\Delta CT}$) of IDO1 transcript in co-injected muscles (BG+IDO1) n=5, **P=0.003.

Figure 5

Inflammatory gene expression changes in IDO1 co-injected muscles. Individual values plot of the relative expression levels ($2^{-\Delta\Delta CT}$) demonstrate significant differences in expression levels of Cd4 (n=4, P=0.018, sidebar=0-6), Cd8 (n=4, P=0.025, sidebar=0-5) and Foxp3 (n=4, P=0.034, sidebar=0-8) in co-injected muscles 14 days post-injection while Il-10 (n=6, P=0.124, sidebar=0.0-2.0) and Il-12 (n=4, P=0.132, sidebar=0.0-3.5) levels were not altered significantly.

Figure 6

Co-expression of IDO1 reduces antibody response against β-gal. The levels of anti-β-gal antibodies 14 days post-injection were significantly lower in sera from mice co-expressing the CMV-driven IDO1 plasmid (P=0.023, ANOVA with Tukey's test). β-gal, n=7; β-gal+CD11c.mIDO1, n=5; β-gal+CMV.mIDO1, n=6.

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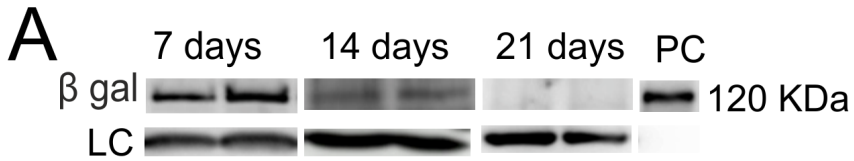
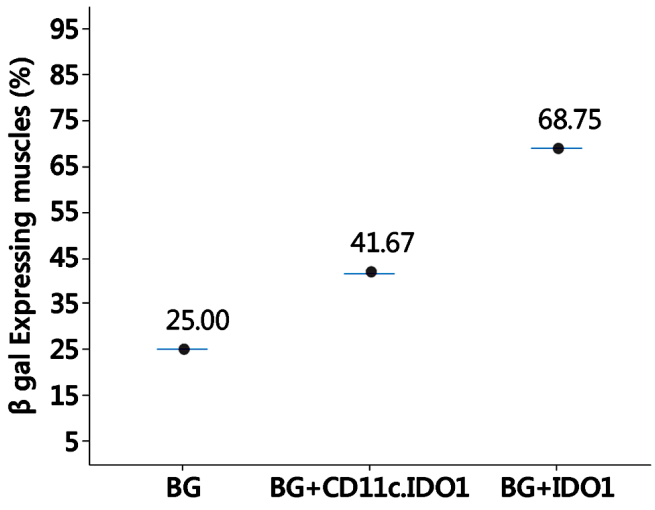
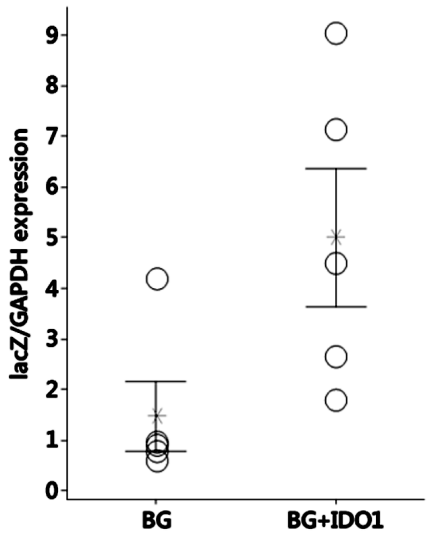
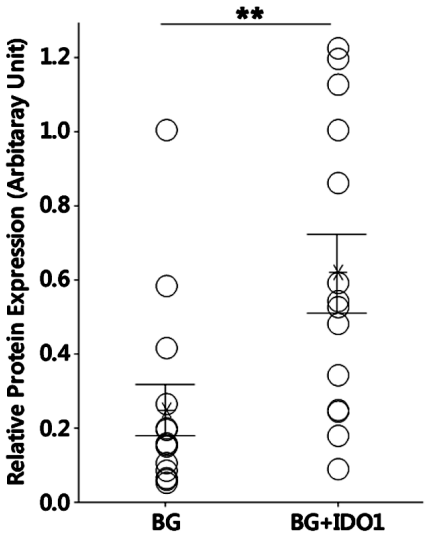
1

2

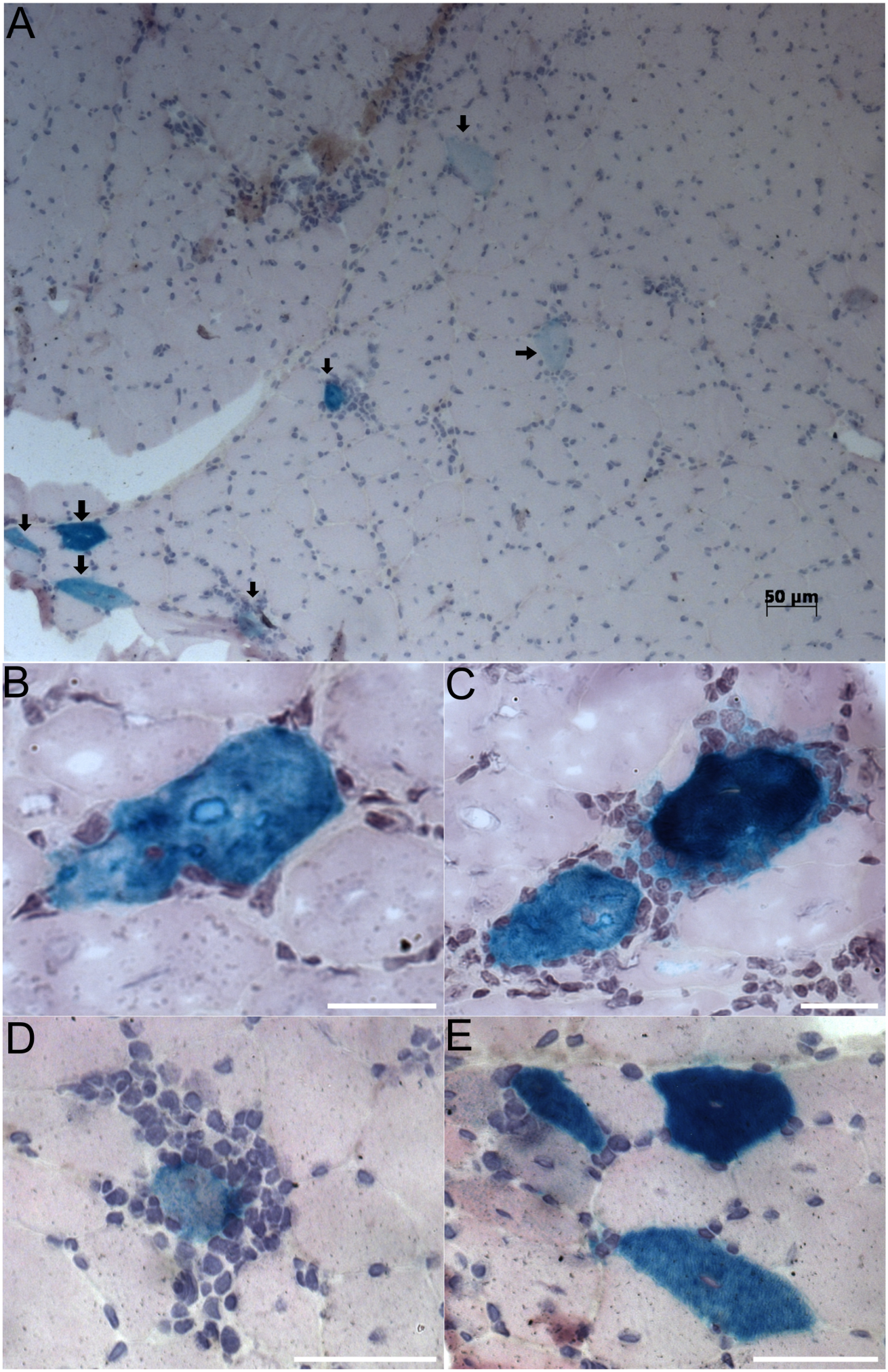

A
7 days
14 days
21 days
PC
β gal
LC
120 KDa


B
β gal Expressing muscles (%)
95
85
75
65
55
45
35
25
15
5
25.00
41.67
68.75
BG
BG+CD11c.IDO1
BG+IDO1


C
lacZ/GAPDH expression
9
8
7
6
5
4
3
2
1
0
BG
BG+IDO1

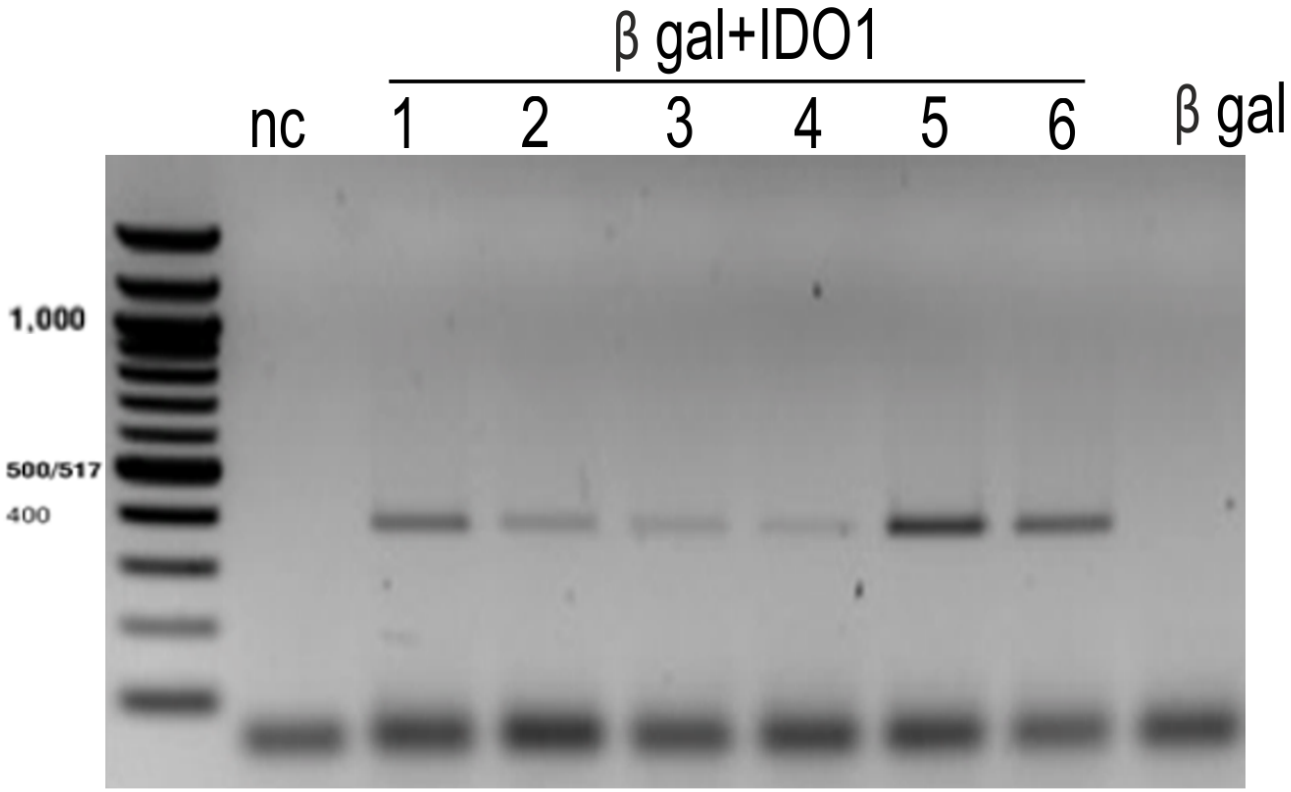

D
**
Relative Protein Expression (Arbitaray Unit)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
BG
BG+IDO1

3

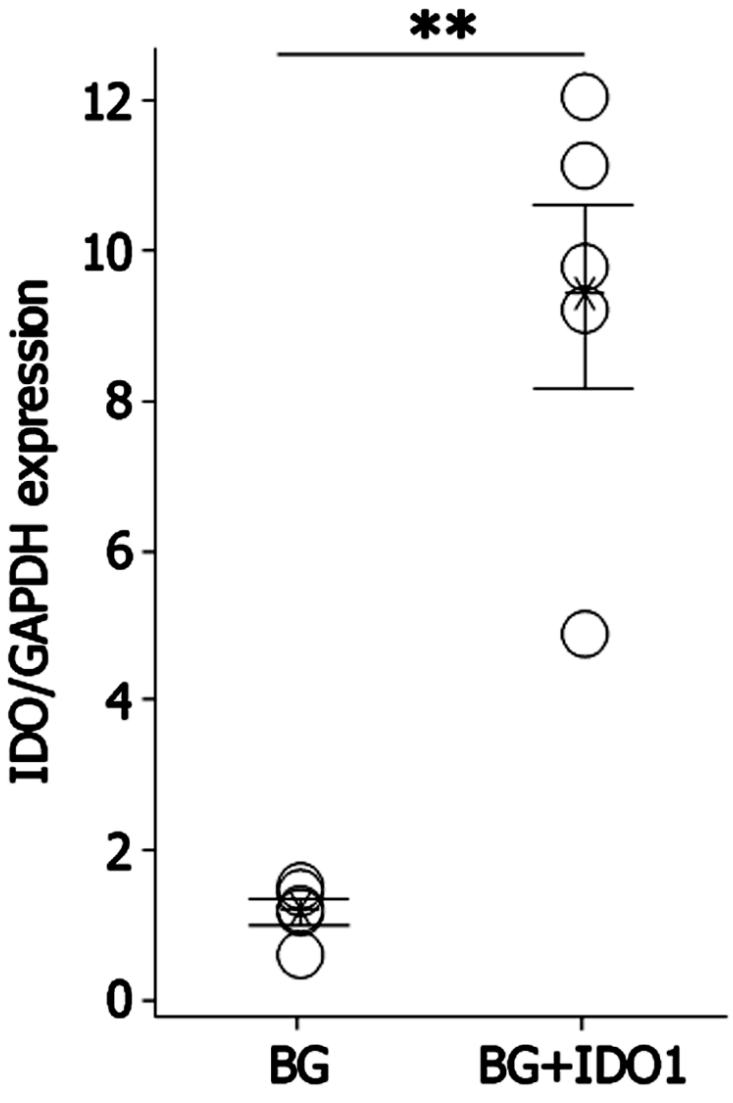


4

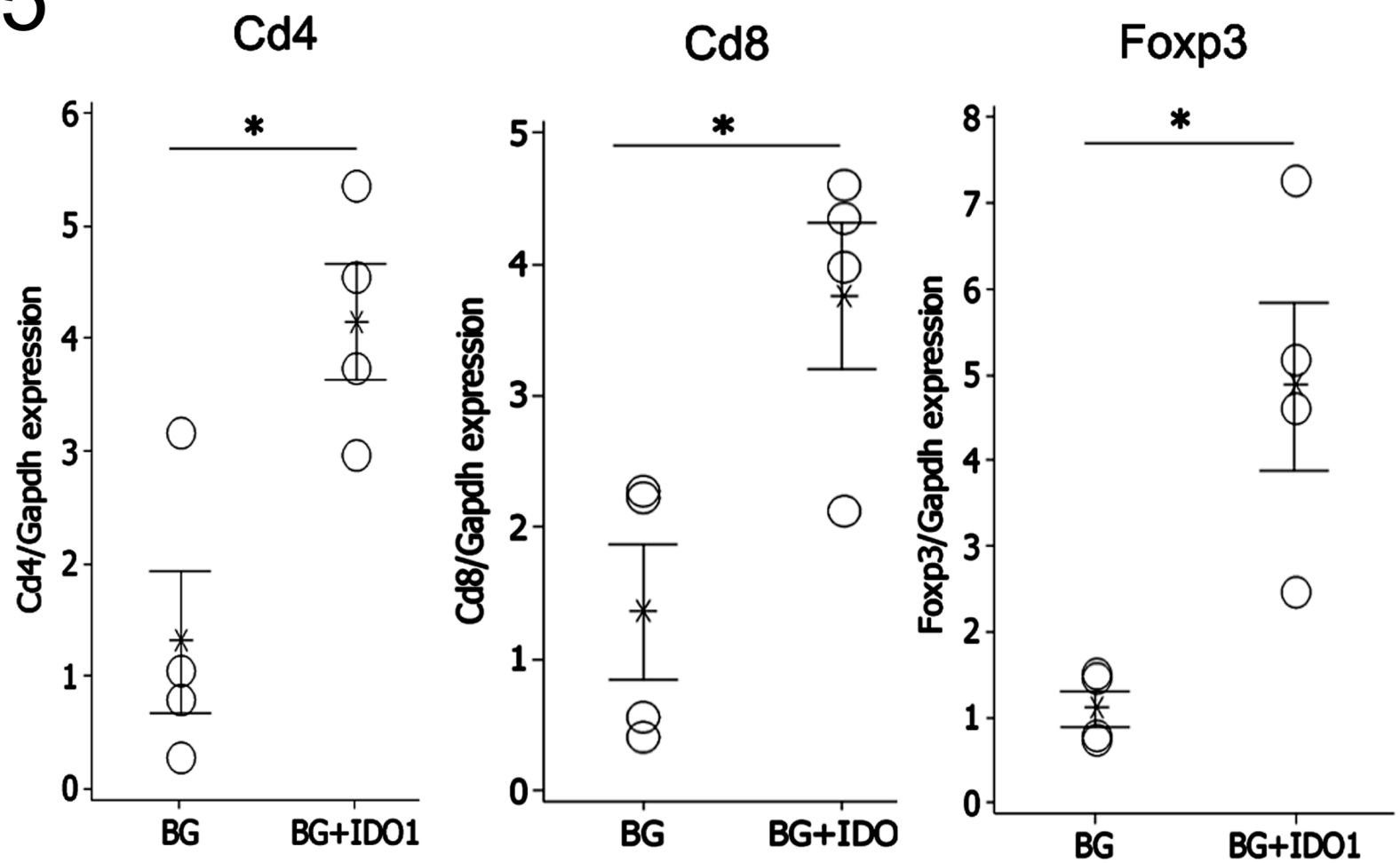
A

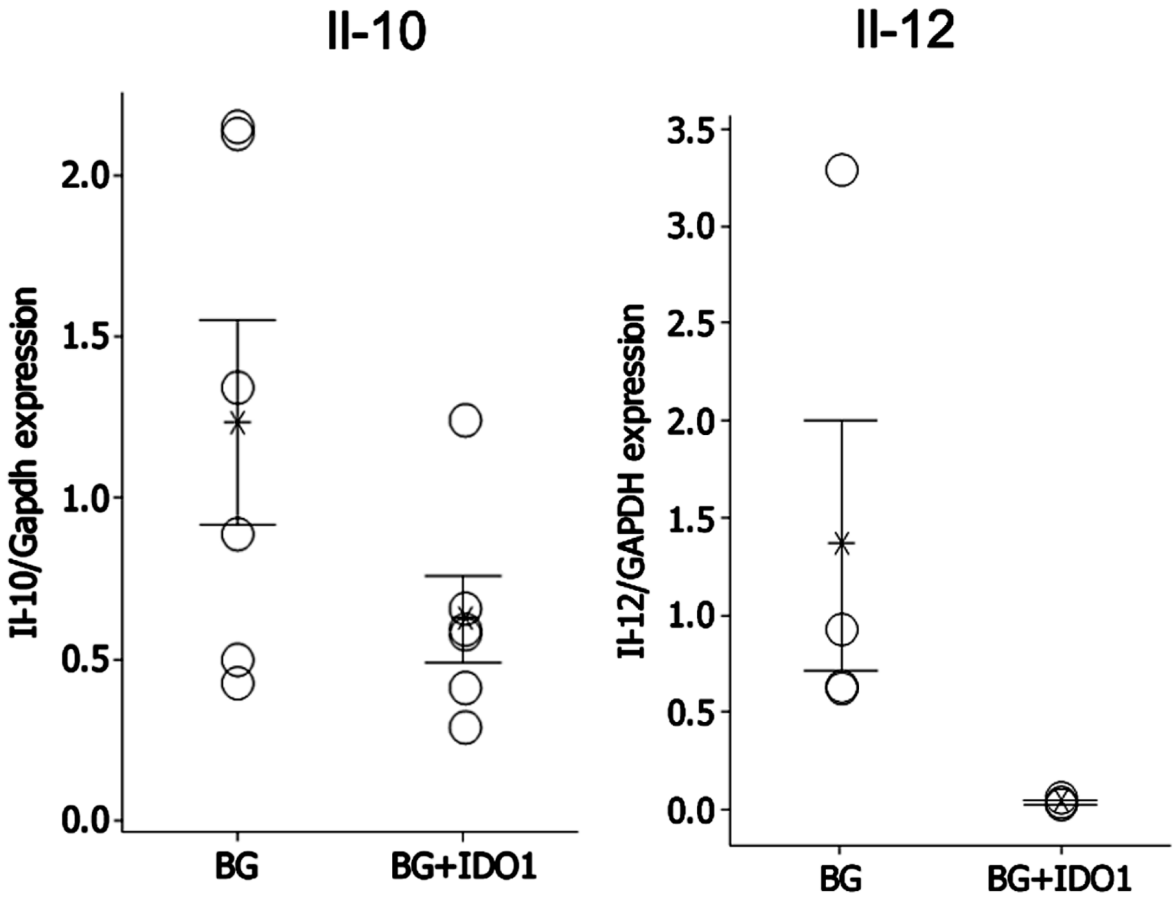

β gal+IDO1
nc
1
2
3
4
5
6
β gal
1,000
500/517
400

B

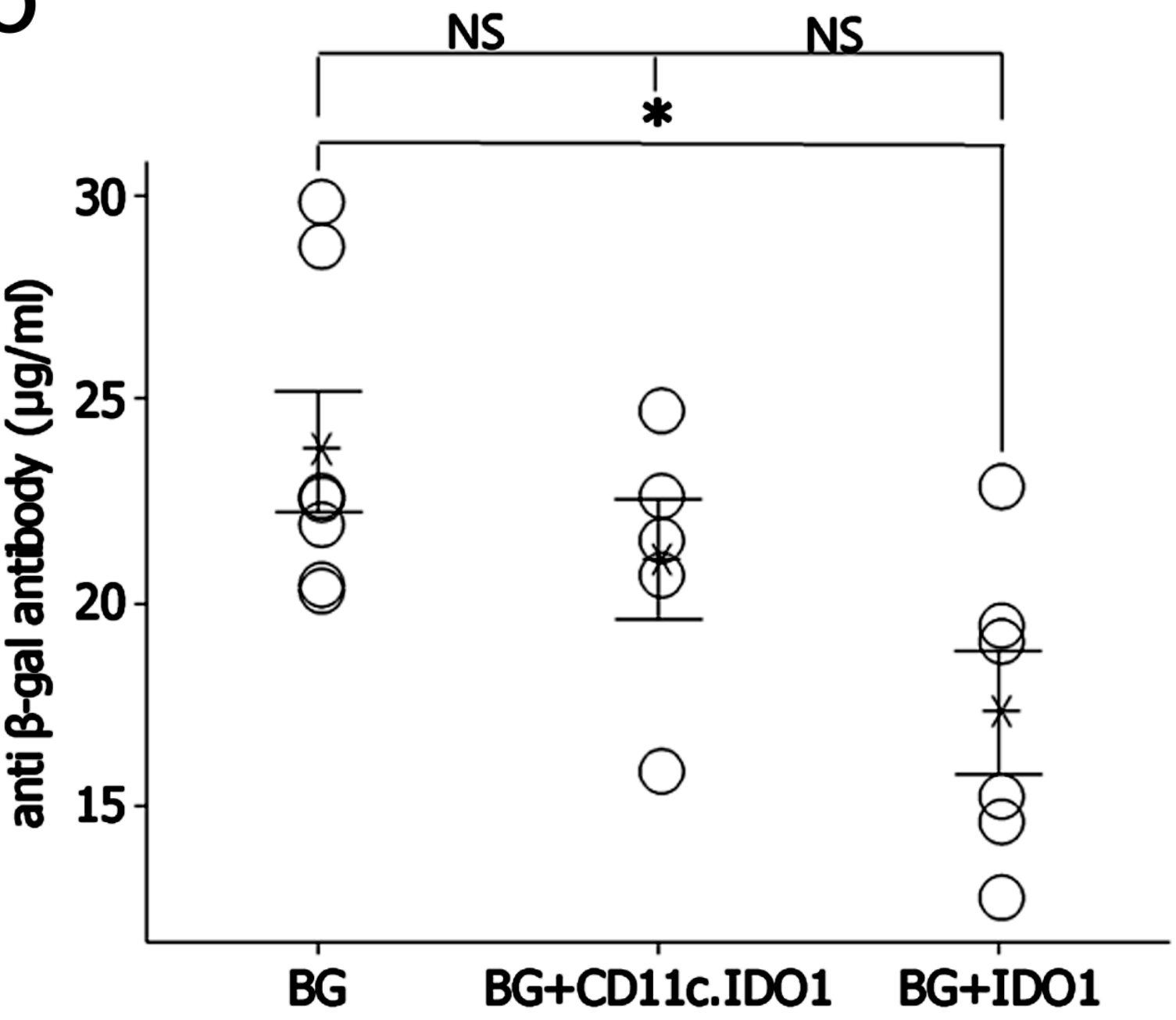

**
IDO/GAPDH expression
12
10
8
6
4
2
0
BG
BG+IDO1

5


Cd4
Cd4/Gapdh expression
*
BG
BG+IDO1
Cd8
Cd8/Gapdh expression
*
BG
BG+IDO
Foxp3
Foxp3/Gapdh expression
*
BG
BG+IDO1


Il-10
Il-10/Gapdh expression
BG
BG+IDO1
Il-12
Il-12/GAPDH expression
BG
BG+IDO1

6


NS
NS
*
30
25
20
15
anti β-gal antibody (µg/ml)
BG
BG+CD11c.IDO1
BG+IDO1